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DESCRIPTION

ENZYME ELECTRODE, SENSOR, FUEL CELL, AND

ELECTROCHEMICAL REACTOR

TECHNICAL FIELD

5   The present invention relates to an enzyme

electrode: more specifically, to an enzyme electrode

comprising a conductive member; and a carrier, an

enzyme, and a mediator immobilized on the conductive

member. The present invention relates also to a

10   sensor, and fuel cell employing the enzyme electrode;

and a process for producing the enzyme electrode.

BACKGROUND ART

       An enzyme, a proteinaceous biocatalyst formed

15   in a living cell, is highly active under mild

conditions in comparison with ordinary catalysts .

Further, the enzyme is. highly specific to a substrate

undergoing an enzymatic reaction, and catalyzes a

specific reaction of a specific substrate. Ideally,

20   the enzyme having such properties will enable

preparation of a highly selective electrode having a

.low overvoltage ,for an oxidation-reduction reaction

on the electrode. However, the active centers of

most redox enzymes (oxidoreductases) are usually

25   enclosed in a deep interior of a three-dimensional

structure of a glycoprotein, so that direct high¬

speed electron transfer cannot be caused between the

oxidoreductase and the electrode. To promote the electron transfer, a method is disclosed which employs a substance called a mediator to connect electronically the enzyme with the electrode. In the

5 early stage of the development, the enzyme and the mediator are dissolved in an electrolyte solution for simplicity of the experiment system and for freedom of motion of the enzyme and the mediator. Later, methods of immobilization thereof on the electrode ,

10 are disclosed for improvement of the efficiency, prevention of leakage from the electrode, and continuous and long-term use of the electrode. The methods for immobilizing the enzyme and the mediator on the conductive member include adsorption,

15 entrapping, crosslinking, and covalent bonding. Of these methods, entrapping, crosslinking, and covalent bonding give high ability for retaining the enzyme and the mediator .and preventing leakage thereof from the system, enabling repeated use of the enzyme

20 electrode in comparison with adsorption methods. Of these methods, in particular, entrapping methods are more advantageous owing to high retention capacity of the enzyme the mediator.

An important index of the performance of the

25 enzyme electrode is an electric current density, ,
which is an electric current intensity for a projected area of a conductive member. The higher

current density enables, for example, improvement in
detection sensitivity, simplification of a
measurement portion, and miniaturization of a sensing
part when used in a sensor based on current intensity
5   detection; improvement of output when used as an
electrode of a fuel cell; and improvement of reaction
velocity when used as an electrochemical reactor,
advantageously. The current density of the enzyme
electrode can be increased by any of the methods of
10   (1) increase of a turnover number (a number of
substrate molecules converted by an enzyme in a unit
time), (2) improvement of the electron transfer rate
and efficiency between the enzyme and the mediator,
(3) improvement of electron transportation in the
15   mediator, (4) improvement of electron transfer rate
and efficiency between the mediator and the
conductive member, (5), increase of the enzyme-holding
density (the amount of the enzyme per projected area
of the conductive member), and so forth.
20   For the increase of the enzyme-holding density
of the above item (5), immobilization methods which
enable high enzyme-retention capacity are
advantageous. Therefore, the above-mentioned
entrapping methods are preferred which entrap the
25   enzyme and the mediator in a carrier and immobilize
the carrier on the surface of the conductive member.
Fig. 1 illustrates schematically a state of

immobilization of an enzyme by an entrapping method.
In Fig. 1, enzyme 1 and mediator 2 are entrapped in
carrier 3 fixed on a conductive member 4 (conducting
substrate) .

5   In the entrapping method, an electric charge
produced on an enzyme by an enzyme/substrate reaction
transfers from oxidation-reduction center of the
enzyme to a mediator in a carrier, and is transported
by electron hopping between the mediator to vicinity
10   of a conductive member, and is finally detected by an
external circuit by transfer of the charge between
the mediator and the conductive member. (In Fig. 1,
the flow of the charge is shown by arrow marks .)
Therefore, even when the enzyme is immobilized in an
15   amount larger than the enzyme full-occupation area
for the effective surface area of the conductive
member, the enzyme immobilization density for the
projected area of the conductive member can.be
improved. For example, an enzyme electrode utilizing
20   the entrapping immobilization for a fuel cell is
disclosed in U.S. Patent 6,531,239 (Heller et al.) in
which the enzyme electrode is prepared by
immobilization of an enzyme by a polymer containing a
mediator in the molecule.

25   Generally, electron transfer velocity between
an enzyme and a mediator is known to decrease
exponentially with increase of the electron transfer

distance between the oxidation-reduction center of
the enzyme and the mediator.   In the entrapping
immobilization,   since the enzyme and the mediator are
not arranged in suitable distances or suitable
5   orientation,   the electron transfer between the enzyme
and the mediator is considered not to occur before
the enzyme and the mediator come close within an
electron-transferable distance by random molecular
motion.   Accordingly,   the electron transfer velocity
10   between the enzyme and the mediator is not sufficient
owing to dependence on the molecular motion of the
both,   and can be a limiting factor of the electric
current density of the enzyme electrode.-

On the other hand,   in an example of the
15   covalent bonding method, a mediator is bonded to a
conducting substrate;   the oxidation-reduction center
of an enzyme is bonded to the mediator;   and an enzyme
excluding the oxidation-reduction center is bonded to
the oxidation-reduction center bonding to the
20   mediator.   Fig. 2 illustrates immobilization state of
an enzyme by a covalent bonding method.   In- this
example of Fig. 2, mediator 6 and enzyme 1 are
immobilized through a carrier substance onto the
surface of conductive member 4 (conducting substrate).
25   In Fig. 2, the flow of charges is shown by arrow
marks .

Kats et al.   (J.Am.Chem.Soc. 1996, vol. 118,

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pp. 10321-10322) discloses an enzyme electrode in
which pyrroloquinolinequinone is bonded to flavin
adenine dinucleotide as a mediator, and apoglucose
oxidase having no oxidation-reduction center is

5   reconstructed thereto. In this enzyme electrode, the
mediator can be arranged near to the oxidation-
reduction center of the enzyme in a distance,
enabling rapid electron transfer with orientation.
In this method, however, the mediator-enzyme pairs

10   are bonded in a monomolecular state onto the
conductive member, and the enzyme molecules cannot be
immobilized in two or more layers, which limits the
enzyme immobilization density and the increase of
electric current density in the enzyme electrode.

15

DISCLOSURE OF THE INVENTION
       The present invention provides an enzyme
electrode which is improved in electric current
density. The present invention provides also a

20 -   sensor, an electrochemical reactor, and a fuel cell
utilizing the enzyme electrode.
       According to an aspect of the present invention,
there is provided an enzyme electrode comprising a
conductive member and an enzyme, wherein a first

25   mediator and a second mediator are immobilized by a
carrier onto the conductive member, the first
mediator and the second mediator having different

redox   potentials   (reduction-oxidation   potentials)   .

     The first mediator preferably has a redox

potential   (reduction-oxidation   potential)   more

negative   than the redox potential   (reduction-

5   oxidation   potential)   of the second mediator,   and the

electron   transfer   reaction   rate between   the second

mediator   and the conductive   member   is higher   than the

electron   transfer   reaction   rate between   the first

mediator   and the conductive   member .

10   The first mediator preferably has. an redox

potential   (reduction-oxidation   potential)   more

positive   than the redox potential   (reduction-

oxidation   potential)   of the second mediator,   and the-

electron   transfer   reaction   rate between   the second

15   mediator   and the conductive   member is higher   than the

electron   transfer   reaction   rate between   the first

mediator   and the conductive   member.

     The first mediator preferably serves to

transfer   electrons   to or from the enzyme,   and the

20   second mediator   serves to transfer   electrons   to or

from the first mediator.

       The conductive   member preferably has a porous

structure.

     The first mediator and the second mediator   are

25   preferably   respectively   at least one substance

selected   from metal complexes,   quinones,   heterocyclic

compounds,   nicotinamide   derivatives,   and flavin

derivatives .

The first mediator preferably has a redox
potential (reduction-oxidation potential) more
negative than the redox potential (reduction-
5   oxidation potential) of the second mediator, and is
employed as an anode .

The second mediator preferably has a redox
potential (reduction-oxidation potential) more
positive than the redox potential (reduction-
10   oxidation potential) of the second mediator, and is
employed as a cathode.

According to another aspect of the present
invention, there is provided a sensor, employing the
enzyme electrode as a detection portion for detecting
15   a substance.

According to still another aspect of the
present invention, there is provided a fuel cell,
employing the enzyme electrode as at least one of
anode and a cathode.

20   According to a further aspect of the present
invention, there is provided an electrochemical
reactor, employing the enzyme electrode as a reaction
electrode.

The process for producing an enzyme electrode
25   having an enzyme immobilized by a carrier on a
conductive member comprises a step of providing a
conductive member, an enzyme, a first mediator for

transfer of electrons to or from the enzyme, a second

mediator for transfer of electrons to or from the

first mediator, and a carrier; and a step of

immobilizing the enzyme, the first mediator, and the

5   second mediator to the conductive member.

According to the present invention, an enzyme

can be immobilized on a conductive member at a high

enzyme-immobilization density relative to the

effective surface area of the conductive member. Use

10   of a first mediator capable of transferring electrons

rapidly to the enzyme, and a second mediator for

charge transport between the first mediator and the

conductive member enables rapid electron transfer to

or from the enzyme, improving the current density in

15   the enzyme electrode.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 is a schematic drawing of entrapping

immobilization in an enzyme electrode.

20   Fig. 2 is a schematic drawing of covalent

bonding immobilization in an enzyme electrode.

Fig. 3 is a schematic drawing of an enzyme

electrode of the present invention.

Fig. 4 is a schematic drawing showing relation

25   of potentials of mediators (in an anode) in the

present invention

Fig. 5 is a schematic drawing showing relation

of potentials of mediators (in a cathode) in the present invention

Fig. 6 is a drawing for explaining a three-electrode cell.

Fig. 7 is a drawing for explaining a two-electrode cell.

Figs. 8A, 8B, 8C and 8D show examples of the porous structures of the conductive members applicable in the present invention.

BEST MODE FOR CARRYING OUT THE INVENTION

Preferred embodiments of the present invention are described below in detail.

The enzyme electrode of the present invention comprises a conductive member and an enzyme, wherein a first mediator and a second mediator are immobilized through a carrier onto the conductive member, the first mediator and the second mediator having different redox potentials (reduction-oxidation potentials).

In a preferred combination of the two mediators, the materials are selected such that the first mediator has a redox potential (reduction-oxidation potential) more negative than the redox potential (reduction-oxidation potential) of the second mediator (as shown in Fig. 4), and the electron transfer reaction rate between the second mediator

and the conductive member is higher than the electron
transfer reaction rate between the first mediator and
the conductive member.

In another preferred combination of the two
5   mediators, the materials are selected such that the
first- mediator has a redox potential (reduction-
oxidation potential) more positive than the redox
potential (reduction-oxidation potential) of the
second mediator (as shown in Fig. 5), and the
10 electron transfer reaction rate between the second
mediator and the conductive member is higher than the
electron transfer reaction rate between the first
mediator, and the conductive member.

The present invention is particularly useful in
15 the cases below.

In one case, a mediator can serve to transfer
electrons to or from an enzyme effectively, but
cannot effectively transfer the electron to or from
the mediator to a conductive member.

20 .   The magnitude relation of electron transfer
rate between a mediator and a conductive member can
be obtained by the comparison of standard rate
constant. This constant is a measure of the kinetic
facility of a redox compounds. Standard rate constant
25 can be estimated from charge-transfer resistance that
is obtained from, for example, impedance measurement.
(Osaka et al. Denkikagakuhou kisosokuteimanual,

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koudansha scientific)

The enzyme electrode of a preferred embodiment
of the present invention comprises an enzyme; at
least one kind of first mediator for transferring an
electron to or from the enzyme; at least one kind of
second mediator for transferring an electron to or
from the first mediator; a carrier immobilizing the
enzyme, the first mediator, and the second mediator;
and a conductive member in contact with the carrier
and transferring the electron to or from the first
mediator or the second mediator. The electron
transfer between the enzyme and the conductive member
is conducted through the first mediator at least and
the second mediator, but the electron transfer may
occur in the absence of the mediators, and/or in the
absence of the second mediatorconductive member.

The use of the carrier enables stable
immobilization of the enzyme on the conductive member,
and increase of the enzyme-holding density for the
effective surface area of the conductive member.
Further, the use of the first mediator capable of
transferring electrons at a high rate to or from the
enzyme and the second mediator capable of
transferring eleótrons between the first mediator and
the conductive member enables electron transfer at a
high rate to or from the enzyme to improve stability
of the electrode and to increase the current density

in the electrode.   The first mediator in the present

invention transfers electrons at a higher rate to or

from the enzyme than the second mediator.

Consequently, the use of the first mediator results

5   in a higher current density in the enzyme electrode

than the one employing the second mediator only

without the first mediator.   On the other hand, the

second mediator in the present invention has higher

charge-transporting ability and/or gives higher

10   electron transfer reaction rate with the electrode

than the first mediator. Consequently, the use of

the second- mediator gives a higher current density in

the enzyme electrode than the one employing the first

mediator only without the second mediator.   The

15   optimum proportions of the enzyme, the first mediator,

the second mediator, and the carrier should be

determined in consideration of the matters ·below: the

proportion of the first mediator should be

sufficiently high for sufficient electron transfer to

20   or from the enzyme; the proportion of the second

mediator should be high for sufficient charge

transport between the enzyme and the conductive

member; the proportion of the carrier is sufficiently

high for sufficient retention of the enzyme and the

25   mediator; and the immobilization density should be

sufficiently high.   For example, for a unit weight of

the enzyme, the first mediator weighs preferably from

0.0001 to 1, more preferably from 0.000.2 to 0.1; the
second mediator weighs preferably from 0.01 to 2,
more preferably from 0.3 to 1; and the carrier weighs
preferably from 0.05 to 3, more preferably from 0.3
5 to 1.

Fig. 3 illustrates an immobilization state of
an enzyme, a first mediator, and a second mediator on
an enzyme electrode of the present invention. In
this example, enzyme 1, first mediator 8, and second
10 mediator 9 are entrapped in layer 3 of a carrier
provided' on the surface of conductive member 4
(conducting substrate) . An example of a flow of
electrons is shown by an arrow mark.

In this enzyme electrode, in use of this enzyme
15 electrode as a positive electrode or a negative
electrode, for effective electron transfer between
the first mediator (for high-speed electron transfer
to or from the enzyme) and the second mediator (for
charge transport between the first mediator and the
20 conductive member) , the first mediator has a redox
potential (reduction-oxidation potential) more
negative than that of the second mediator when the
enzyme electrode is utilized as an anode (Fig. 4),
whereas the first mediator has a redox potential
25 (reduction-oxidation potential) more positive than
that of the second mediator when the enzyme electrode
is utilized as a cathode (Fig. 5).

A sensor, which is a preferred embodiment of
the present invention, employs the enzyme electrode
as a sensing part for detecting a substance. In a
typical constitution, an enzyme electrode as a
5   working electrode, a reference electrode, and a
counter electrode are employed in a set. A catalytic
current is detected by the enzyme electrode (by
function of the enzyme immobilized on the electrode)
to detect a substance in the liquid in contact with
10   the electrodes. Fig. 6 shows a specific example of
the constitution of the sensor. In Fig. 6, the
sensor comprises anode 15, platinum wire 16, and
optionally Ag/AgCl reference electrode 17, and the
respective electrodes are connected through lead
15   wires 18-20 to a potentiostat . This sensor is placed
in electrolyte solution 14 in water-jacketed cell 12
closable with a cover 13. A substrate in the
electrolyte solution can be detected by applying a
potential to the working electrode and measuring the
20   steady-state current. When the measurement should be
conducted in an inert atmosphere, an inert gas like
nitrogen is introduced from gas inlet 22 of gas tube
23. The temperature can be controlled by a
temperature-controlling liquid by introduction of
25   controlling liquid inlet 24 to temperature-
controlling liquid outlet 25.

The constitution of the sensor of the present

invention is not limited insofar as the enzyme   · -
electrode can conduct detection. This sensor has
high substrate selectivity owing to the enzyme
employed as the electrode reaction catalyst, and has

5   high stability and high current density owing to use
of the carrier and plural mediators, whereby the
sensor can be used repeatedly for a long term, the
sensor can be simplified, or the sensing part can be
miniaturized.   The enzyme electrode may be in a board

10 shape, or a single or multiple layer shape. This
sensor is capable of detecting a substance
corresponding to the substrate of the enzyme of the
enzyme electrode, being useful, for example, as a
glucose sensor, a fructose sensor, a galactose sensor,

15 an amino acid sensor, an amine sensor, a cholesterol
sensor, an alcohol sensor, a lactic acid sensor, an
oxygen sensor, a hydrogen peroxide sensor, or the
like. More specific application examples are a
sensor for measuring a glucose concentration or

20 lactic acid concentration in blood, a sensor for
measuring a sugar concentration in a fruit, and a
sensor for measuring an alcohol concentration in
exhaled breath.

      A fuel cell, a preferred embodiment of the

25 present invention, employs an enzyme electrode as at
least one of the anode and the cathode . In this
embodiment also, the enzyme electrode may be in a

board shape, a monolayer shape, or a multilayer
structure. In the multilayer structure, anodes and
cathodes are arranged as predetermined. In a typical
constitution, the fuel cell has a reaction layer for
5   holding an electrolyte solution containing a fuel
source material, and an anode and a cathode placed at
a predetermined interval, at least one of the anode
and the cathode being the enzyme electrode of the
present invention. The fuel cell may be of a type in
10   which an electrolyte solution is replenished or
circulated, or may be of a type in which an
electrolyte solution is neither replenished nor
circulated. The fuel cell is not limited in the fuel,
the structure, the function, and so forth, insofar as
15   the enzyme electrode is usable. The enzyme, the
catalyst constituting the enzyme electrode, has a
high substrate selectivity in comparison with a noble
metal catalyst (e.g., platinum) employed generally in
electrochemical fields. Therefore, the reaction
20   substances on the anode and the cathode need not be
separated by a partition, which can simplify the
device. This fuel cell can generate a high driving
voltage by oxidation-reduction of a substance at a
low overvoltage owing to a characteristic high
25   activity of the employed enzyme as the catalyst for
the electrode reaction. The fuel cell can have a
long life and give high output by using a carrier and

plural mediator.

Fig. 7 shows an example of the fuel cell. The fuel cell has a constitution similar to the one shown in Fig. 6 of the substrate detecting sensor.

5   Therefore, in Figs. 6 and I_1 the same reference numerals are used for denoting the correspondent conductive members . The fuel cell is constituted by replacing the sensor in Fig. 6 by an electrode unit constituted of anode 15 and cathode 27 laminated with

10   interposition of a porous polypropylene film. The fuel cell is driven by introducing gaseous oxygen through tube 23 into the cell.

An electrochemical reactor, a preferred embodiment of the present invention, can give high

15   substrate selectivity and high catalytic ability characteristic of the enzyme utilized in the electrode reaction catalyst as well as the quantitativeness characteristic to the electrochemical reaction. Therefore, the reactor is

20   capable of controlling the reaction quantitatively with high selectivity, and high efficiency. The reactor can have a long life and a high power output with high stability and a high current density by use of a carrier and plural mediators. In this

25   embodiment also, the enzyme electrode may be in a board shape, a monolayer shape, or a multilayer structure. In a typical constitution, a pair of

electrodes and an optionally provided reference
electrode are placed in a reaction vessel, and an
electric current is allowed to flow between the pair
of electrodes to cause an electrochemical reaction of
5   a substance in the reaction solution to obtain an
intended reaction product or decomposition product:
at le.ast one of the pair of electrodes is an enzyme
electrode of the present invention. The kind of the
reaction solution, the reaction conditions, and the
10   constitution of the reactor are not specially limited,
insofar as the enzyme electrode is usable. For
example, the reactor is useful for obtaining an
intended reaction product or decomposition product by
an oxidation-reduction reaction.
15   The electrochemical reactor can cause reaction
of a substance corresponding to the substrate of the
enzyme used in the enzyme electrode. For example,
the reactor is useful for oxidation of glucose,
fructose, galactose, amino acids, amines, cholesterol,
20   alcohols, lactic acid, and so forth; reduction of
oxygen, hydrogen peroxide, and so forth; and the like
reactions. More specific application examples
include selective oxidation of cholesterol in the
presence of ethanol, and reduction of oxygen at a low
25   overvoltage.
         The carrier in the present invention serves at
least to immobilize the enzyme to the conductive

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member. The carrier includes (1) polymer compounds,
(2) inorganic compounds, and (3) organic compounds,
the compounds having a covalent bonding site in the
molecule and being capable of bonding an enzyme to
5   the conductive member (the carrier may have
additionally a function of bonding two enzymes) . The
carrier contains at least one of the above three
types of compounds. To immobilize the enzyme to the
electrode, the carrier has preferably an electric
10   charge opposite to the surface charge of the enzyme
under the electrode driving conditions . The carrier
may be ones capable of holding the enzyme by covalent
bonding, electrostatic interaction, spatial trapping,
or a like action to hold the enzyme stably at a high
15   density in comparison with retention of the enzyme by
physical adsorption to the electrode or to a binder
polymer for caking the electrode .
        The polymer compounds useful as the carrier
include electroconductive polymers such as
20. polyacetylenes, polyarylenes, polyarylene-vinylenes,
polyacenes, polyarylacetylenes, polydiacetylenes,
polynaphthalenes, polypyrroles, polyanilines,
polythiophenes , polythienylene-vinylenes ,
polyazulenes, and polyisothianaphthenes; and other
25   kind of polymers such as polystyrenesulfonic acids,
polyvinyl sulfate, dextran sulfate, chondroitin
sulfate, polyacrylic acid, polymethacrylic acid,

polymaleic acid, polyfumaric acid, polyethylenimine,
polyallylamine hydrochloride,
polydiallyldimethylammoniur α chloride ,
polyvinylpyridine, polyvinylimidazole, polylysine,

5   deoxyribonucleic acid, ribonucleic acid, pectin,
silicone resins, cellulose, agarose, dextran, chitin,
polystyrene, polyvinyl alcohol, and nylons.

The inorganic compounds useful as the carrier
include metal chalcogenide compounds containing at

10   least one element selected from the group of In, Sn,
Zn, Ti, Al, Si, Zr, Nb, Mg, Ba, Mo, W, V, and Sr.

The organic compounds, being useful as the
carrier, and having a covalent bonding site in the
molecule and being capable of bonding an enzyme to

15   the conductive member, and/or the two enzymes include
compounds having at least one functional group
selected from hydroxyl, carboxyl, amino, aldehydo,
hydrazino, thiocyanato, epoxy, vinyl, halogeno, acid
ester groups, phosphato, thiol, disulfido,

20   dithiocarbamato, dithiophosphato, dithiophosphnato,
thioether groups, thiosulfato, and thiourea groups.
Typical examples are glutaraldehyde, polyethylene
glycol diglycidyl ether, cyanuric chloride, N-
hydroxysuccinimide esters, dimethyl-3, 3·-

25   dithiopropionimidate hydrochloride, 3,3'-dithio-
bis (sulfosuccinimidyl propionate) , cystamine, alkyl
dithiols, biphenylene dithiols, and benzene dithiols .

The conductive member in the present invention serves to hold the enzyme, the mediator, and the carrier for immobilizing the enzyme and the mediators, and to connect the enzyme electrode electrically to
5   an external circuit on driving the enzyme electrode. This conductive member should have electroconductivity, sufficient rigidity during storage and measurement, and should be electrochemically stable under electrode driving
10 conditions. The constituting material of the conductive member includes electroconductive materials such as metals, electroconductive polymers, metal oxides, and carbonaceous materials.

The metal includes those containing at least
15 one element of Au, Pt, Ag, Co, Pd, Rh, Ir, Ru, Os, Re, Ni, Cr, Fe, Mo, Ti, Al, Cu, V, .Nb, Zr, Sn, In, Ga, Mg, Pb, Si, and W . The metal may be an alloy, or a plating metal.

The electroconductive polymers include those
20 containing at least one of polyacetylenes, polyarylenes, polyarylene-vinylenes, polyacenes, polyarylacetylenes , polydiacetylenes , polynaphthalenes, polypyrroles, polyanilines, polythiophenes, polythienylenevinylenes, polyazulenes,
25 and polyisothianaphthenes .

The metal oxides may contain other conductive material for improving the conductivity thereof or

imparting conductivity. The metal oxides include oxides of metals containing at least one of In, Sn, Zn, Ti, Al, Si, Zr, Nb, Mg, Ba, Mo, W, V, and Sr. The electroconductive materials include metals electroconductive polymers, and carbonaceous materials. The carbonaceous material may contain other electroconductive material for improving or imparting the electroconductivity. The carbonaceous materials include graphite, carbon black, carbon nanotubes, carbon nanohorns, fullerene compounds, and derivatives thereof.

The electric conductivity of the conductive member applicable to the present invention ranges from 0.1 to 700000 S/cm, preferably from 1 to 100000 s/cm, more preferably from 100 to 100000 S/cm. (Incidentally, "S" denotes Siemens, a reciprocal of ohm $(1/\Omega)$.) Even when the enzyme electrode has a porous structure, the conductive member has preferably the electric conductivity in the above range.

The conductive member has preferably a structure having numerous voids communicating with each other, the voids communicating with the outside of the conductive member directly or through other voids and being capable of immobilizing an enzyme, a first mediator, and a second mediator therein. The conductive member having numerous voids communicating

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with the outside has preferably a structure in which
void-partitioning walls are formed integrally from
the constitution material, or the void-partitioning
walls are bonded tightly with each other. The

5   material for constituting the conductive member
includes the above-mentioned conductive materials of
metals, polymers, metal oxides, and carbonaceous
substances .

The numerous voids in the conductive member are

10   interconnected together in one-, two-, or three-
dimensionally. The interconnection of the voids may
be of two or more types. The one-dimensional void
interconnection is exemplified by columnar voids; the
two-dimensional void interconnection is exemplified

15   by net-like voids; and the three-dimensional void
interconnection is exemplified by sponge-like voids,
interstices formed in aggregation of small particles,
and voids, in a structural material prepared by use of
the above material as a template. The voids size

20   should be large for introduction of the enzyme and
flow and diffusion of the substrate substance, but
should be small within the range for obtaining a
sufficient ratio of the effective void surface area
to the projected area of the conductive member. The

25   average void size ranges, for example, from 5 nm to
500 μm, more preferably from 10 nm to 100 μm. The
void-containing conductive member should have a small

thickness for introduction of the enzyme into the
deep interior of the conductive member and flow and
diffusion of the substrate through the conductive
member, but should have thickness within the range

5   for obtaining a sufficient ratio of the effective
void surface area to the projection area of the
conductive member. The thickness of the void-
containing conductive member ranges, for example,
from 100 nm to 1 cm, more preferably from 1 μm to 5

10 mm. The ratio of the effective surface area to the
projected area of the void-containing conductive
member should be sufficiently large, for example, the
ratio being 10 or more, more preferably 100 or more.
The porosity of the void-containing conductive member

15 should be sufficiently large for obtaining a high
ratio of the effective void surface area to the
projected area of the conductive member, and be large
within the range for enabling introduction of the
enzyme and the carrier and flow and diffusion of the

20 substrate substance, but should be small for
achieving the sufficient mechanical strength. The
porosity ranges,- for example, from 20% to 99%, more
preferably from 30% to 98%. The porosity of the
conductive member having an enzyme immobilized

25 therein should be large for flow of the electrolyte
solution and diffusion of the substrate substance,
but should be small by filling of the enzyme. The

porosity ranges for example, from 15% to 98%, more

preferably from 25% to 95%.

The constituting material for the void-
containing conductive member includes foamed metals,

5   electrodeposited metals, electrolytic metals,

sintered metals, fibrous metals, and metals

corresponding to two or more of the above kinds of

metals .

The void-containing polymer can be produced by

10   any of the processes for manufacture of a porous

resin. In one process, a conductive polymer is

molded in the presence of a void template into an

intended shape, and thereafter the material of the

void template is removed. In another process, a

15   template for the voids is placed in a prepolymer, the

prepolymer is polymerized into a conductive polymer,

and thereafter the material of the template is

removed. In a still another process, a layer is

formed from particles for constituting a void

20   template, a polymer is filled into the interstice of

the particle layer, and thereafter the particles are

removed from the layer. In a still another process,

a layer is formed from particles for constituting a

void template, a prepolymer is. filled into the

25   interstice of the particle layer, the prepolymer is

polymerized to form a polymer layer, and thereafter

the particles are removed from the layer.

The void-containing conductive member constituted of a metal oxide having numerous voids can be obtained by a method including electro-deposition, sputtering, sintering, chemical vapor

5   deposition (CVD), electrolysis, and so forth, and combinations thereof. Otherwise, the conductive member having numerous voids can be obtained from fibers or particles of graphite, carbon black, carbon nanotubes, carbon nanohorns, a fullerene compound, or

10   a derivatives thereof by forming the material into an intended shape and sintering the formed material.

In the conductive member, the voids may have a Uniform size (or uniform porosity) in the thickness direction of the porous structure, or the voids may

15   have a gradient distribution of the size or porosity.

Figs . 8A to 8D illustrates porous structures of the conductive member., In the drawings, the numerals denote the followings : 801, an electrolyte layer; 802, a pore; 803, a conductive member; 804, supporting

20   substrate optionally employed. As shown in. the drawings, the sizes of the pores in the conductive member are preferably larger at the electrolyte layer side and smaller in the inside.

In other words, in the conductive porous

25   conductive member employed in the present invention, the pore sizes are preferably larger at the surface side of the conductive porous conductive member than

those at the interior thereof. The pore size ratio

is preferably 2 or more, more preferably 4 or more,

still more preferably 10 or more, but is not larger

than 1000.

5   The porosity may be the same between the

regions of different pore sizes. More preferably,

the pore sizes and the porosities in the conductive

member are both larger in the electrolyte layer side,

and smaller in the interior.

10   The pore size and porosity of the porous

conductive member can be measured by nitrogen gas

adsorption measurement (BET method (Brunauer-Emmett-

Teller method)), for example by AUTOSORB-I

(Quantachrome Instruments Co.) . The pore sizes on

15 the surface of the conductive member can be estimated

by measuring the pore sizes of a certain number of

pores (e.g., about 50-300 pores) in SEM photograph

(scanning electron microscope. photograph)..

The mediator in the present invention promotes

20 electron transfer through enzyme/mediator/conductive

member. This mediator may be chemically bonded to

the carrier and/or the enzyme. The mediator is

exemplified by metal complexes, quinones,

heterocyclic compounds, nicotinamide derivatives, and

25 flavin derivatives. The metal complexes include

those having as the central metal at least one

element selected from Os, Fe, Ru, Co, Cu, Ni, V, Mo,

Cr, Mn, Pt, Rh, Pd, Mg, Ca, Sr, Ba, Ti, Ir, Zn, Cd,
Hg, and W . The ligands of the metal complexes are
exemplified by those containing an atom of nitrogen,
oxygen, phosphorus, sulfur, or carbon and capable of

5   forming a complex through the above atom with the
central metal; and those having a cyclopentadienyl
ring as the skeleton. The ligand includes pyrrole,
pyrazole, imidazole, 1,2,3- or 1,2,4-triazole,
tetrazole, 2,2'-biimidazole, pyridine, 2,2'-

10 bithiophene, 2,2'-bipyridine, 2,2':6'2"-terpyridine,
ethylenediamine, porphyrin, phthalocyanine,
acetylacetone, quinolinol, ammonia, cyan ,ions,
triphenylphosphine oxide, and derivatives thereof.
The quinones as the mediator include quinone,

15 benzoquinone, anthraquinone, naphthoquinone,
pyrroloquinolinequinone (PQQ) ,
tetracyanoquinonedimethane, and derivatives thereof.
The heterocyclic compounds as the mediator include
phenazine, phenothiazine, biologen, and derivatives

20 thereof. The nicotinamide derivatives as the
mediator include nicotinamide adenine dinucleotide
(NAD) , and nicotinamide adenine dinucleotide
phosphate. The flavin derivatives as the mediator
include flavin adenine dinucleotide (FAD) .

25   The enzyme in the present invention serves to
catalyze an oxidation-reduction reaction, and may be
a combination of plural enzymes . The enzymes include

glucose oxidase, galactose oxidase, bilirubin oxidase, pyruvate oxidase, D- or L-amino acid oxidase, amine oxidase, cholesterol oxidase, choline oxidase, xanthine oxidase, sarcosine oxidase, L-lactate
5   oxidase, ascorbate oxidase, cytochrome oxidase, alcohol dehydrogenase, glutamate dehydrogenase, cholesterol dehydrogenase, aldehyde dehydrogenase, glucose dehydrogenase, fructose dehydrogenase, sorbitol dehydrogenase, lactate dehydrogenase, malate.
10 dehydrogenase, glycerol dehydrogenase, 17B-hydroxysteroid dehydrogenase, estradiol-17B dehydrogenase, amino acid dehydrogenase, glyceraldehyde-3-phoshate dehydrogenase, 3-hydroxysteroid dehydrogenase, diaphorase, catalase, .
15 peroxidase, glutathione reductase, NADH-cytochrome b5 reductase, NADPH-adrenodoxin reductase, cytochrome b5 reductase, adrenodoxin, reductase, and nitrate reductase.

      The substrate substances for the electrode-
20 constituting enzymes are compounds corresponding to the respective enzymes, including organic matters, oxygen, hydrogen peroxide, water, and nitrate ions. The organic matters include sugars, alcohols, carboxylic acids, quinones, nicotinamide derivatives,
25 and flavin derivatives. The sugars include polysaccharides such as cellulose, and starch.

      In the step of immobilizing the carrier in the

present invention, uniformity is important in the

immobilization of the carrier on the conductive

member. In the step of immobilizing the carrier, the

enzyme and/or the mediator may be concurrently

5   immobilized. The step of immobilizing the carrier

may be a step of immersing a void-containing

conductive member in a solution or dispersion liquid

of the carrier; a step of applying, injecting, or

spraying a solution or dispersion liquid of the

10   carrier onto a void-containing conductive member; or

a step of applying, injecting, dr spraying a solution

or dispersion liquid of the carrier precursor onto a

void-containing conductive member and thereafter

hydrolyzing, polymerizing, or crosslinking the

15   precursor for immobilization.

     In the drawings, the reference numerals denote

the followings : 1, an enzyme; 2 and 6, a mediator; 3

and 7, a carrier; 4, a conductive member; 5, a flow

of a charge; 8, a first mediator; 9, a second

20   mediator; 10, a flow of an electron; 11, a potential

(the arrow .indicates the negative direction); 12, a

water-jacketed cell; 13, a cover for the water-

jecketed cell; 14, an electrolysis solution; 15, an

anode; 16, a platinum wire; 17, an Ag/AgCl reference

25   cell; 18, an anode lead; 18, a cathode lead; 20 a

reference electrode lead; 21, a potentiostat; 22, a

gas inlet; 23, a gas tube; 24, a temperature-

controlling water inlet; 25, a temperature-

controlling outlet; 26, porous polypropylene film; 27,

a cathode .

Examples.   "

5   The present invention is explained below in

more detail by reference to examples.without limiting

the invention.

        Firstly, the processes for preparation of the

conductive members for use for the enzyme electrode

10   of the present invention are described.

    (Preparation Example 1)

        A commercial silica colloid dispersion liquid

    (Nissan Chemical Ind.; average particle size: 100 nm)

    is employed. The dispersion medium of the dispersion

15   liquid is replaced by ethanol. A cleaned gold

    substrate (1 cm square., 0.3 mm thick, Nilaco) is

    allowed to stand in the dispersion liquid. The

    ethanol is allowed to evaporate a,t 30°C to obtain a

    porous film constituted of silica spheres . This

20   process is repeated several times to increase the

    thickness of a porous film constituted of silica

    spheres (100 nm thick) . The film is heated at 200 °C

    for three hours, and then washed with ethanol. In a

    three-electrode cell, by use of this porous film as

25   the working electrode, a platinum electrode as the

    counter electrode, and an Ag/AgCl electrode as the

    reference electrode, electrolytic polymerization is

conducted in a solution of 0.IM 3,4-

ethylenedioxythiophene and 0.IM lithium perchlorate

in acetonitrile at a potential of 1.1 V (vs Ag/AgCl)

by control with a potentiostat . The time of the

5   polymerization is controlled by monitoring the

electrolysis current profile to obtain a film in a

thickness nearly equivalent to the silica sphere

porous film thickness. After the electrolytic

polymerization, the film is immersed in a 20%

10   hydrofluoric acid solution for two days to remove the

silica spheres to obtain a conductive member (100 μm

thick) constituted of electroconductive poly(3,4-

ethylenedioxythiphene) containing numerous voids.

(Preparation Example 2).

15   Needle-shaped indium tin oxide (ITO, Sumitomo

Metal Mining Co.; length: 30-100 run; aspect ratio: 10

or higher) is dispersed in terpinol, and the

viscosity is adjusted by addition of ethylcellulose

to obtain an ITO paste. This ITO paste is applied on

20   a cleaned gold substrate (1 cm square, 0.3 mm thick,

Nilaco) by screen process printing, and is sintered

at 450 °C for one hour to obtain- a porous ITO sintered

electrode (100 μm thick). Further thereon, ITO is

deposited by plasma chemical vapor phase deposition

25   (CVD) in a thickness of about 10 nm to obtain a

conductive member constituted of ITO having numerous

voids .

(Preparation Example 3)

Natural particulate graphite (particle size: 11 μm) is mixed with polyvinylidene fluoride in an amount of 10 wt% of the particulate graphite. N-
5   methyl-2-pyrrolidone is added thereto to solve the polyvinylidene fluoride. The blended graphite paste is molded into a sheet of 11.3 mm diameter and 0.5 mm thick. The film is dried at 60°c, heated to 240°C, and further vacuum-dried at 200°C. Thereby a
10 conductive member is obtained which is constituted of many graphite particles bonded together and has interconnected numerous voids in the structure.

(Preparation Example 4)

N[6]-(2-aminoethyl) FAD shown by Chemical Formula
15 (1) is prepared through the process shown below. To an aqueous 10% FAD solution, is added an equimolar amount of ethylenimine , The pH is adjusted to 6-6.5. The mi.xtμre is allowed to react at 50°C for 6 hours. The reaction solution is cooled, and is added into
20 ethanol on an ice bath to cause precipitation. The precipitate is collected and is purified by anion exchange chromatography and reversed-phase high-speed chromatography to obtain purified N[6]-(2-aminoethyl) FAD shown by Chemical Formula (1).

· ·. · · (1)
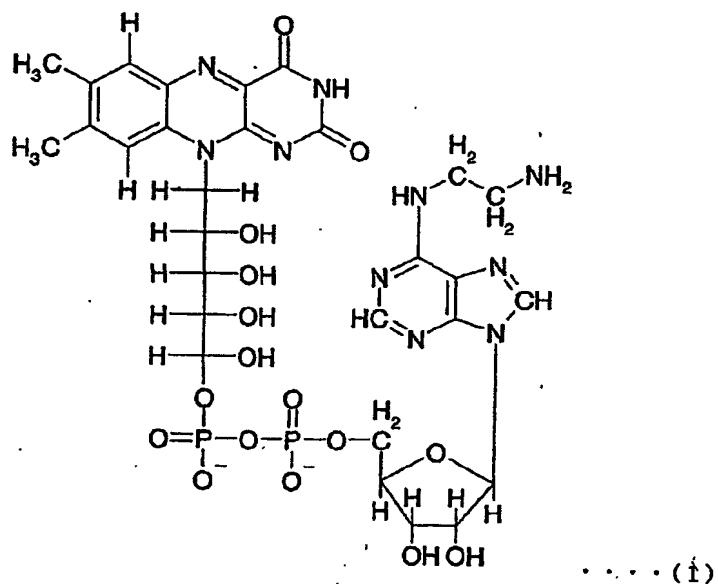
(Preparation Example 5)

The process for synthesis of the ferrocene

5   derivative shown by Chemical Formula (2) is described
below. A 4.7 g portion of β-aminocaproic acid is
dissolved in 200 mL of ,DMF. Thereto, is added a
solution of 2.1 g ferrocene carbaldehyde in 100 mL of
DMF. The mixture is stirred at 100 °C for one hour.

10   Thereto is added 1 g of sodium boron hydride
saturated in water. The mixture is stirred at room
temperature for one hour. The solvent is evaporated
off under a reduced pressure. The evaporation
residue is treated by a silica column with a solvent

15   of dichloromethane/methanol (10/1) to obtain the
ferrocene derivative compound represented by Chemical
Formula (2) . The compound is identified by ^{1}H-NMR.
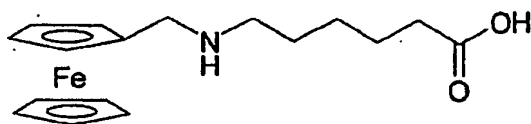
· · · · -(2)

(Preparation   Example   6)

      The   osmium   complex-containing.   polymer   shown   by-

Chemical   Formula   (3)   is   synthesized   through   the   steps

5   below.   To   6 irlL   of   1-viylimidazole,   is   added   0.50   g

of   azobisisobutyronitrile   .   The   mixture   is   allowed   to

react   in   an   argon   atmosphere   at   70°C   for   2   hours.

After   air-cooling   of   the   reaction   mixture,   the   formed

precipitate   ·is   dissolved   in   methanol,   and   the   ·

10   solution   is   added   dropwise   to   acetone   with   violent

agitation.   The   formed   precipitate   is   collected   by

filtration   to   obtain   poly-1-vinylimidazole   .

Separately,   to   1.2   g   of   4,4'-dimethyl-2,   2'-bipyridine

and   1.4   g   of   ammonium   hexachloroosmate,   is   added   5 mL

15   of   ethylene   glycol.   The   mixture   is   allowed   to   react

in   a   nitrogen   atmosphere   at   140 °C   for   -24   hours.   The

reaction   solution   is   cooled   by   air,   and   is   filtered

to   remove   impurity.   The   filtrate   is   evaporated   at a

reduced   pressure   to   obtain   Os (4, 4'-dimethyl-2,   2'-

20   bipyridine)   2 dichloride   salt.   To   200   mL   of   ethanol,

are   added   0.13   g   of   Os (4, 4'-dimethyl-2,   2'-

bipyridine)   2 dichloride   salt   and   0.20   g   of

polyvinylimidazole.   The   mixture   is   refluxed   in   a

nitrogen   atmosphere   for 3 days . The   reaction

solution   is filtered,   and the   filtrate   is added

dropwise   to 1 L of diethyl   ether   with   violent

agitation.   The   formed   precipitate   is recovered   and

5   dried   to obtain   the osmium   complex   polymer   shown   by

Chemical   Formula   (3) . The   compound   is identified   by

elemental   analysis.
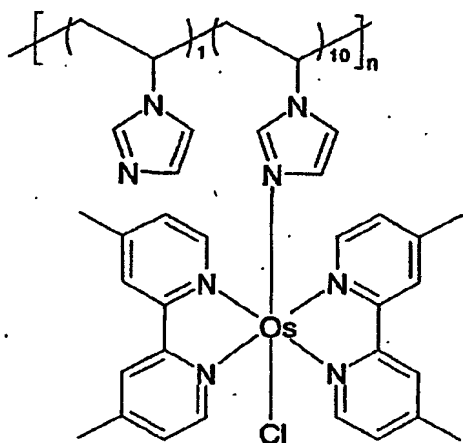
- (3)

10   (Preparation   Example   7)

The   ferrocene-containing   polymer   shown   by

Chemical   Formula   (4) is prepared   through   the 'steps

below.   To 3.0 rtⅰL of toluene,   are added   1.6 g of 1-

vinylimidazole,   0.71   g of 1-vinylferrocene,   and 6.2

15   mg of azobisisobutyronitrile   . The mixture   is allowed

to react   in an argon   atmosphere   at 70 °C for 2 hours.

The reaction   solution,   after   air-cooling,   is added

dropwise   to 1 L o-f diethyl   ether.   The   formed

precipitate is collected by filtration to obtain the
ferrocene-containing polymer shown by Chemical
formula (4) .
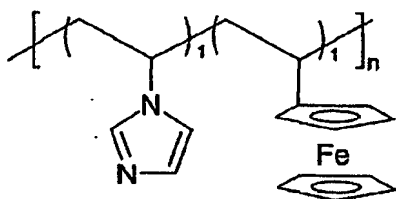
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5   (Preparation Example 8)

The process for synthesis of the complex
polymer shown by Chemical Formula (5) is described
below.

To 100 g of an aqueous 40% glyoxal solution, is
10   added dropwise 370 mL of an aqueous concentrated
ammonia solution on an ice bath. The mixture is
stirred at 45°C for 24 hours, and is air-cooled. The
formed precipitate is collected by filtration, and
vacuum-dried at 50°C for 24 hours to obtain 2,2'-
15   biimidazole. This compound is identified by silica-
gel thin-layer chromatography (methanol/chloroform
(10%/90%)). To a solution of 4.6 g of 2,2'-
biimidazole in 100 mL of N,N'-dimethylformamide (DMF),
is added 2.7 g of sodium hydride in a nitrogen
20   atmosphere on an ice bath. The mixture is stirred at
room temperature for one hour. Thereto, a solution
of 12.8 g of methyl p-toluenesulfonate in 5 mL of DMF

is added dropwise in 20 minutes, and the mixture is
stirred at room temperature for 4 hours. The solvent
is evaporated under vacuum at 50°C. The evaporation
residue is washed with 50 mL of hexane, and vacuum-
5   dried at 160°C to obtain N,N'-dimethyl-2, 2'-
biimidazole in a colorless transparent crystalline
state. The obtained product is identified by ^{1}H-NMR.

To a solution of 10 g 2,2'-biimidazole in 100-
mL of DMF, is added 3.3 g of sodium hydride on an ice
10   bath in a nitrogen atmosphere. The mixture is
stirred on an ice bath for one hour. Thereto, 4.6 mL
of methyl iodide is added dropwise, and the mixture
is stirred on an ice bath for 30 minutes and at room
temperature for 12 hours . The reaction solution is
15   poured into 300 mL of ethyl acetate. The mixture is
filtered, and the solvent is evaporated from the
filtrate under a reduced pressure and vacuum. The
evaporation residue is dissolved in boiling ethyl
acetate., and the solution is filtered. The filtered
20   ethyl acetate solution is refluxed again. Thereto
300 mL of hexane is added for saturation. The
solution is kept in a refrigerator for 12 hours for
crystal growth. The crystalline matter is collected
by suction filtration, and recrystallized from ethyl.
25   acetate/hexane to obtain N-methyl-2, 2'-biimidazole .
This compound is identified by ^{1}H-NMR.

A I g portion of N-methyl-2, 2'-biimidazole is

dissolved in 80 mL of DMF. Thereto, 0.32 g of sodium

hydride is added in a nitrogen atmosphere. The

mixture is stirred on an ice bath for one hour.

Thereto 2.5 g of N-(β-bromohexyl)phthalimide and 1.0

5   g of sodium iodide are added gradually. The mixture

is stirred in a nitrogen atmosphere at 80°C for 24

hours. The mixture is cooled to room temperature,

and 150 mL of water is added thereto. The mixture is

extracted twice with 150 mL portions of ethyl acetate.

10 The ethyl acetate solution is washed with an aqueous

sodium chloride solution and dried over sodium

sulfate, and is evaporated under a reduced pressure.

The residue is purified by a neutral alumina column

(ethyl acetate/hexane 10-40%) to obtain N-methyl-N'-

15 (β-phthalimidohexyl) -2,2'-biimidazole . This product

is identified by ¹-H-NMR.

A 2.5 g portion o,f N-methyl-N' - (6-

phthalimidohexyl) -2,2'-biimidazole is dissolved in 25

mL of ethanol. Thereto 0.39 g of hydrogenated

20 hydrazine is added. The mixture is refluxed for 2

hours, cooled to room temperature, and filtered. The

solution is transferred to a silica gel column with

ethanol. The product is recovered by a 10% ammonia

solution in acetonitrile, and the solution is

25 .evaporated under a reduced pressure to obtain N-(6-

aminohexyl) -N'-methyl-2,2'-biimidazole. This product

is identified by ¹H-NMR.

In 40 mL of ethylene glycol, 1.1 g of N-methyl-2,2'-biimidazole and 1.4 g of ammonium hexachloroosmate are dissolved. The solution is stirred in a nitrogen atmosphere at 140 °C for 24

5 hours. Thereto, is added a solution of 0.8 g of N-(6-aminohexyl)-N'-methyl-2,2'-biimidazole in 5 mL of ethylene glycol. The. solution is stirred further for 24 hours, cooled to room temperature, and filtered. The filtrate is diluted with 200 mL of water, and

10 stirred with 40 mL of an anion exchange resin (DOWEX® 1X4) in the atmosphere for 24 hours. The solution is poured gradually into a solution of 10.2 g of ammonium hexaf luorophosphate in 150 mL of water. The precipitate is collected by filtration by suction,

15 and dissolved in acetonitrile and reprecipitated by an aqueous ammonium hexaf luorophosphate solution. The obtained matter" is washed with water, and vacuum-dried- at 45 °C for 24 hours to obtain osmium (III) (N, N'-dimethyl-2, 2'-biimidazole) 2 (N- (6-aminohexyl) -

20 N'-methyl-2, 2'-biimidazole) hexaf luorophosphate salt. This product is identified by elemental analysis.

To 150 mL of DMF, are added 20 g of polyvinylpyridine (average molecular weight: 150,000) and 5.6 g of 6-bromohexane . The mixture is stirred

25 at 90 °C with a stirrer for 24 hours, and cooled to room temperature. The cooled mixture is poured gradually into 1.2 L of ethyl acetate with violent

agitation. Then the solvent is removed by
decantation, and the remaining solid matter is
dissolved in methanol. The solution is filtered, and
evaporated to a solvent volume of about 200 inL. The

5   formed product is reprecipitated with 1 L of diethyl
ether. The product is vacuum-dried at 50°C for 24
hours, pulverized, and further dried for 48 hours to
obtain poly (4- (N- (5-carboxypentyl)pyridinium) -co-4-
vinylpyridine) .

10   In 10 mL of DMF, 0.52 g of the poly (4- (N- (5-
carboxypentyl) pyridinium) -co-4-vinylpyridine) is
dispersed, and thereto 0.18 g of O- (N-succinimidyl) -
N ,N ,N ' ,N ^{r} -tetramethyluronium tetraflu óroborate (TSTU)
is added. The mixture is stirred ·for 15 minutes.

15   Thereto 0.1 mL of N ,N-diisopropylethylamine is added,
and the mixture is stirred for 8 hours. Thereto,
0.89 g of poly (4- (N- (5rcarboxypentyl) pyridinium) -co-
4-vinylpyridine) is added and the mixture is stirred
for 5 minutes. Further thereto, 0.1 mL of N ,N -·

20   diisopropylethylamine is added and the mixture is
stirred at room temperature for 24 hours. The
resulting mixture is added to 200 mL of ethyl acetate..
The formed precipitate is collected by filtration,
and is added to 30 mL of acetonitrile . Thereto 40 mL

25   of DOWEX® 1X4, and 100 mL of water are added, and the
mixture is stirred for 36 hours to dissolve the
polymer. The solution is filtered by suction, and is

43

concentrated to a volume of 50 mL. The concentrated
matter is extruded through a (mol wt 10000) -cutoff
filter (Millipore) at a nitrogen pressure of '275 kPa.
Further, the extruded matter is passed with water as
5   the solvent through a DOWEX® 1X4 column, and dialyzed
in water. Thereby the polymer (chloride salt) of
Chemical Formula (1) is obtained.
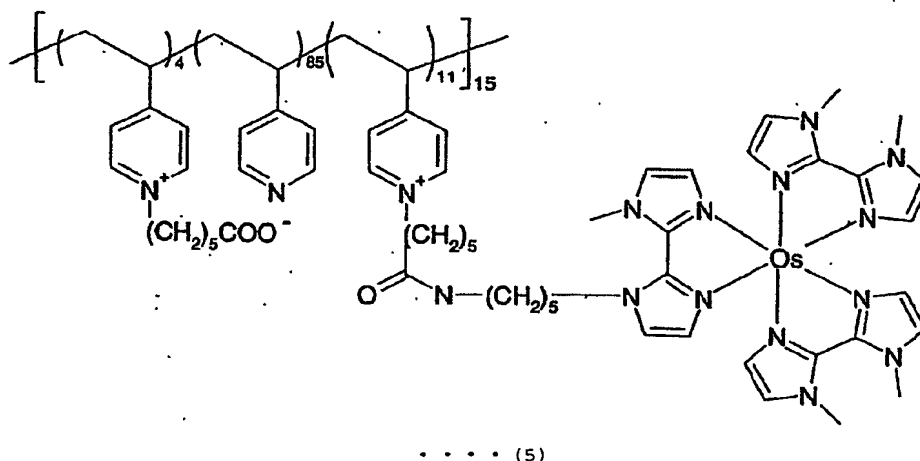
· · · · (5)

(Preparation Example 9)
10   The process for synthesis of the complex
polymer shown by Chemical Formula (6) is described
below.

To 6 mL of 1-vinylimidazole, is added 0.5 g of
azobisisobutylonitrile . The mixture is allowed to
15   react i-n \an argon atmosphere at 70°C for 2 hours.
The reaction solution is ai-r-cooled. The formed
precipitate is dissolved in methanol. The solution
is added dropwise into acetone with violent agitation..

The precipitate is collected by filtration to obtain
poly-1-vinylimidazole.   Separately,   0.76 g of
2,2':6'2"-terpyridine   and 1.42 g of ammonium
hexachloroosmate   are added to 5 mL of ethylene glycol,
5   and the mixture is refluxed in an argon atmosphere
for one hour. To this solution, 0.60 g of 4,4'-
dimethyl-2, 2'-bipyridine is added. The mixture is
refluxed for 24 hours. The reaction solution is air-
cooled.   Impurity is removed by filtration.   The
10 filtrate is evaporated to remove the- solvent to
obtain osmium (2, 2':6'2"-terpyridine) (4, 4'-dimethyl-
2,2'-bipyridine) chloride salt.

A 200 mL portion of ethanol is added to 0.38 g
of osmium(2,2' :6'2"-terpyridine) (4, 4'-dimethyl-2, 2'-
15 bipyridine) chloride salt and 0.2 g of
polyvinylimidazole . The mixture is refluxed in a
nitrogen atmosphere for three days . The reaction
mixture is filtered, and then the filtrate is added
dropwise into 1 L of diethyl ether with violent
20 agitation.   The formed precipitate is recovered and
dried to obtain the osmium complex represented by
Chemical Formula (6) . The compound is identified by
elemental analysis.
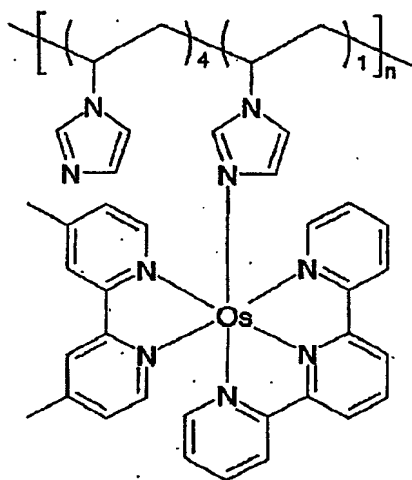
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(Preparation Example 10)

An FAD-free apoglucose oxidase is prepared through the process below. Glucose oxidase

5   (Aspergillus niger) is dissolved in 3 mL of a 0.25M sodium phosphate buffer solution (pH 6) containing 30% glycerol., This solution is cooled to 0^0c, and the pH thereof is adjusted to 1.7 by addition of a 0.025M sodium phosphate buffer solution-sulfuric acid

10   solution containing 30% glycerol (pH 1.1). This solution is allowed to pass through a Sephadex® G-25 column with a 0.IM sodium phosphate solution (pH: 1.7) containing 30% glycerol, and the intended fraction is recovered by monitoring with light of a

15   wavelength of 280 nm. Dextran-coated charcoal is added to the recovered solution. The solution, after adjustment of pH to 7 by addition of a IM sodium

hydroxide solution, is stirred at 4°C for one hour. The resulting solution is centrifuged, passed through a 0.45μm filter, and dialyzed by use of a 0.IM sodium phosphate buffer solution to obtain the apoglucose

5   oxidase.

(Preparation Example 11)

An FAD-free apo-D-amino acid oxidase is prepared through the process below.- D-amino acid oxidase (swine kidney) is dialyzed against a 0.IM

10   pyrophosphate buffer solution containing IM potassium bromide and 3.OmM ethylenediamine tetraacetate at 4°C for 2 days with four liquid-exchange's. After confirmation of disappearance of the color of holo-D-amino acid oxidase, the solution is dialyzed against

15   a 0.lM pyrophosphate buffer solution (pH 8.5) for 2 days with three liquid-exchanges to remove potassium bromide to obtain apo-D-amino acid oxidase.

(Preparation Example 12)

A PQQ-modified FAD is prepared through the

20   process below. A 10 inM portion of PQQ is added to a 0.01M HEPES buffer solution (pH 7.3) containing 1 mM of N^6-(2-aminoethyl) FAD described in Preparation Example- 4 and 10 mM of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide . The mixture is

25   stirred for 2 hours, and ultrafiltered to eliminate excessive PQQ to obtain PQQ-modified FAD.

(Preparation Example 13)

A ferrocene-modified FAD is prepared in the
same manner as in Preparation Example 12 except that
the ferrocene derivative described in Preparation
Example 5 is used instead of PQQ.

5   (Preparation Example 14)

A PQQ-modified glucose oxidase is prepared
through the process below. To a solution of 4 mg/mL
of apoglucose oxidase prepared in Preparation Example
10 in a 0.IM phosphate buffer solution (pH 7.0), is

10   added 0.2 mg/mL of the PQQ-modified FAD described in
Preparation Example 12. The mixture is stirred at
25°C for 4 hours and at 4°C for 12 hours, and
ultrafiltered to eliminate excessive PQQ to obtain
PQQ-modified glucose oxidase.

15   (Preparation Example 15)

A ferrocene-modified glucose oxidase is
prepared in the same manner as in Preparation Example
14 except that the ferrocene derivative obtained in
Preparation Example 13 is used instead of PQQ-

20 · modified FAD.

(Preparation Example 16)

A ferrocene-modified D-amino acid oxidase is
prepared in the same manner as in Preparation Example
15 except that the apo-amino acid oxidase described

25   in Preparation Example 11 is used instead of apo¬
glucose oxidase described in Preparation Example 10
described in Preparation Example 14 described in

Preparation Example 15.

(Preparation Example 17)

An enzyme electrode for cathode of a fuel cell is prepared as described below. In a sample tube,

5   the osmium complex polymer described in Preparation Example 9 is dissolved in 5 mL of water at a concentration of 10 mg/mL. Thereto, are added 1 mL of 0.2M citrate buffer solution (pH 5) and 1 mL of an aqueous 30mg/mL solution of laccase (Coriolus

10   hirsutus). After stirring, thereto is added 2 mL of aqueous 10mg/mL polyethylene glycol diglycidyl ether solution. The mixture is stirred. Separately, a sheet of a nickel alloy (Mitsubishi Materials Corp.; constituting elements: Ni, Cr, Ti, Nb,- Al, Mn,- Si,

15   and C; 0.5 mm thick; plated with 0.5μm thick gold; pore size: 50 μm) is cut in 1 cm square, washed, and subjected to UV-ozone treatment. This foamed nickel al-loy sheet is immersed in the above-prepared enzyme-osmium polymer solution, then taken out and dried in

20   a desiccator for 2 days to prepare an enzyme electrode.

The processes for preparation of enzyme electrodes of the present invention are described below.

25   (Example 1)

In a sample tube, 1 mL of an aqueous solution containing 0.1 M sodium hydrogencarbonate and 40

mg/mL of the PQQ-modif ied glucose oxidase described

in Preparation Example 14 is prepared.   Thereto, is

added 0.5 mL of an aqueous 7mg/mL sodium periodate

solution.   The mixture is stirred in the dark for one

5   hour.   Thereto are added 6 mL of aqueous 10%

polypyrrole solution and a mixture of 0.4 mL of an

aqueous 2 .5mg/mL polyethylene glycol diglycidyl ether

solution and 0.42 g of potassium

hexacyanof errate (III) . . Separately, a gold sheet (1

10   cm square, 0.3 mm thick, .Nilaco) is cleaned and

subjected to UV-ozone treatment. This gold sheet is

immersed in the above-prepared mixture solution, then

taken out and dried in a desiccator for 2 days to

prepare an enzyme electrode.

15   (Example 2)

        An enzyme electrode is prepared in the same

manner as in Example 1, except that a 10% polyaniline

solution is used instead of the 10% polypyrrole

solution, and 0.63 g of octacyanotungstate (IV) is

20   used instead of 0.42 g of potassium

hexacyanof errate (III) .

    (Example 3)

        In a sample tube, 1 mL of an aqueous solution

containing 0.1 M sodium hydrogencarbonate and 40

25   mg/mL of the PQQ-modified glucose oxidase described

, in Preparation Example 14 is prepared.   Thereto, is

added 0.5 mL of an aqueous .7mg/mL sodium periodate

solution.   The   mixture   is stirred   in the   dark   for   one

hour.   Thereto   are added   6 ᵢᵣᵢₗ of aqueous   10mg/mL

solution   of the osmium   complex   polymer   described   in

Preparation   Example   6 and 0.4   mL of an aqueous

5   2.5mg/mL   polyethylene   glycol   diglycidyl   ether

solution.   Separately,   a gold sheet   (1 cm square,   0.3

mm thick,   Nilaco)   is cleaned   and subjected   to UV-

ozone   treatment.   This gold sheet   is immersed   in the

above-prepared   mixture   solution,   then   taken   out and

10   dried   in a desiccator   for 2 days   to prepare   an enzyme

electrode.

(Example   4)

In a sample   tube,   1 mL of an aqueous   solution

containing   0.1 M sodium   hydrogencarbonate   and 40

15   mg/mL   of the PQQ-modified   glucose   oxidase   described

in Preparation   Example   14 is prepared.   Thereto,   is

added   0.5 mL of an aqueous   7mg/mL sodium   periodate

solution.   The mixture   is stirred   in the dark   for one

hour.   Thereto   are added   6 mL of aqueous   4mg/mL

20   solution   of polyvinylimidazole   described   in

Preparation   Example   6 and a mixture   of 0.4 mL of an

aqueous   2.5mg/mL   polyethylene   glycol   diglycidyl   ether

solution   and 0.42   g of potassium

hexacyanof   errate   (III). Separately,   a gold sheet   (1

25   cm square,   0.3 mm thick,   Nilaco)   is cleaned   and

subjected   to UV-ozone   treatment.   This gold sheet   is

immersed   in the above-prepared   mixture   solution,   then

taken out and dried in a desiccator for 2 days to prepare an enzyme electrode.

(Example 5)

An enzyme electrode is prepared in the same manner as in Example 4 except that 0.63 g of potassium octacyanotungstate (IV) is used in stead of o."42 g of potassium hexacyanoferrate (III) .

(Example 6)

An enzyme electrode is prepared in the same manner as in Example 5 except that the ferrocene-modified glucose oxidase described in Preparation Example 15 is used instead of PQQ-modified glucose oxidase.

(Example 7)

An enzyme electrode is prepared in the same manner as in Example 6 except that the ferrocene-modified amino acid oxidase described in Preparation Example 16 is used instead of ferrocene-modified glucose oxidase.

(Example 8)

An enzyme electrode is prepared in the same manner as in Example 4 except that polyvinylpyridine (av Biol wt: 150,000) is used instead of the polyvinylimidazole described in Preparation Example 6.

(Example 9)

An enzyme electrode is prepared in the same manner as in Example 5 except that poly-L-lysine (ay

mol wt: 150,000) is used instead of the
polyvinylimidazole described in Preparation Example 6.

(Example 10)

An enzyme electrode is prepared in the same

5   manner as in Example 3 except that ferrocene-
containing polymer described in Preparation Example 7
is used instead of the osmium complex polymer
described in preparation Example 6.

(Example 11)

10   An enzyme electrode is prepared in the same
manner as in Example 1 except that foamed nickel
alloy (Mitsubishi Materials Corp.; constituting
elements: Ni, Cr, Ti, Nb, Al, Mn, Si, and C; 0.5 mm
thick; plated with 0.5 μm thick gold; pore size: 50

15   μm) is used instead of the gold sheet.

(Example 12)

An enzyme electrode is prepared in the same
manner as in Example 2 except that foamed nickel
alloy (Mitsubishi Materials Corp.; constituting

20   elements: Ni, Cr, Ti, Nb, Al, Mn, Si, and C; 0.5 mm
thick-; plated with 0.5 μm thick gold; pore size: 50
μm) is used instead of the gold sheet.

(Example 13)

An enzyme- electrode is prepared in the same

25   manner as in Example 3 except that foamed nickel
alloy (Mitsubishi Materials Corp.; constituting
elements: Ni, Cr, Ti, Nb, Al, Mn, Si, and C; 0.5 mm

thick; plated with 0.5 µm thick gold; pore size: 50
µm) is used instead of the gold sheet.

(Example 14)

An enzyme electrode is prepared in the same

5   manner as in Example 4 except that foamed nickel
alloy (Mitsubishi Materials Corp.; constituting
elements: Ni, Cr, Ti, Nb, Al, Mn, Si, and C; 0.5 mm
.thick; plated with 0.5 µm thick gold; pore size: 50
µm) is used instead of the gold sheet.

10   (Example 15)

An enzyme electrode is prepared in the same
manner as in Example 5 except that foamed nickel
alloy (Mitsubishi Materials Corp.; constituting
elements: Ni, Cr, Ti, Nb, Al, Mn, Si, and C; 0.5 mm

15   thick; plated with 0.5 µm thick gold; pore size: 50
µm) is used instead of the gold sheet.

(Example 16)

An enzyme electrode is prepared in the same
manner as in Example 6 except that foamed nickel

20   alloy (Mitsubishi Materials Corp.; constituting
elements: Ni, Cr, Ti, Nb, Al, Mn, Si, and C; 0.5 mm
thick; plated with 0.5 µm thick .gold; pore size: 50
µm) is used instead of the gold sheet.

(Example 17)

25   An enzyme electrode is prepared in the same
manner as in Example 7 except that foamed nickel
alloy (Mitsubishi Materials Corp.; constituting

elements: Ni, Cr, Ti, Nb, Al, Mn, Si, and C; 0.5 mm
thick; plated with 0.5μm thick gold; pore size: 50
μm) is used instead of the gold sheet.

(Example 18)

5   An enzyme electrode is prepared in the same
manner as in Example 8 except that foamed nickel
alloy (Mitsubishi Materials Corp.; constituting
elements: Ni, Cr, Ti, Nb, Al, Mn, Si, and C; 0.5 mm
thick; plated with 0.5μm thick gold; pore size: 50
·10   μm) is used instead of the gold sheet.

(Example 19)

          An enzyme electrode is prepared in the same
manner as in Example 9 except that foamed nickel
alloy (Mitsubishi Materials Corp.-; constituting
15   elements: Ni, Cr, Ti, Nb, Al, Mn, Si, and C; 0.5 mm
·thick; plated with 0.5μm thick gold; pore size: 50
μm) is used instead of, the gold sheet.

(Example 20)

          An enzyme electrode is prepared in the same
20   manner as in Example 10 except that foamed nickel·
alloy (Mitsubishi Materials Corp.; constituting
elements: Ni, Cr, Ti, Nb, Al, Mn, Si, and C; 0.5 mm
thick; plated with 0.5μm thick gold; pore size: 50
μm) is used instead of the gold sheet.

25   (Example 21)

          An enzyme electrode is prepared in the same
manner as in Example 3 except that the conductive

member constituted of poly (3, 4-ethylenedioxythiphene) containing numerous voids described in Preparation Example 1 is used instead of the gold sheet.

(Example 22)

5   An enzyme electrode is prepared in the same manner as in Example 3 except that the conductive member constituted of ITO described in Preparation Example 2 is used instead of the gold sheet.

(Example 23)

10   An enzyme electrode is prepared in the same manner as in Example 3 except that a transparent -conductive glass sheet constituted of fluorine-doped tin oxide (Asahi. Glass Co.; 8Ω/sq) is used instead of the gold sheet.'

15 (Example 24)

       An enzyme electrode is prepared in the same manner as in Example 3 -except that the conductive member constituted of graphite having numerous voids described in Preparation Example 3 is used instead of

20 the gold sheet.

(Comparative Example 1)

       A gold sheet (1 cm square; 0.3 mm thick; Nilaco) is cleaned and is subjected to UV-ozone treatment. This gold sheet is immersed in an aqueous

25 0.02M cystamine solution for 2 hours, then taken out, and washed with water to prepare a cystamine-modif ied conductive member. This conductive member is

immersed in a 0.01M HEPES buffer solution containing
3 mM of PQQ and 10 inM of 1-ethyl-3- (3-
dimethylaminopropyl) carbodiimide for one hour, and
washed with water for PQQ modification of the

5   conductive member. This PQQ-modified conductive
member is immersed in a 0.01M HEPES buffer solution
containing ImM N^5 (2-aminoethyl) FAD described in
Preparation Example 4 and 10mM 1-ethyl-3- (3-
dimethylaminopropyl) carbodiimide for 2 hours and

10   washed with water for FAD modification of the
conductive member. Further, this conductive member
is immersed in a 0.IM phosphate buffer solution (pH
7.0) containing .4mg/mL apoglucose oxidase described
in Preparation Example 10 at 25°C for 4 hours and at

15   4°C for 12 hours, then taken out, and further
immersed in a phosphate buffer solution for one hour
to prepare an enzyme electrode .
(Comparative Example 2)

An enzyme electrode is prepared in the same

20- manner as in Example 1 except that the potassium
hexacyanoferrate (III) is not added to the enzyme
solution.
(Comparative Example 3)

An enzyme electrode is prepared in the. same

25   manner as in Example 1 except that glucose oxidase
(Aspergillus niger) is used instead of the PQQ-
modified glucose oxidase described in Preparation

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Example 14;

(Comparative Example 4)

An enzyme electrode is prepared in the same
manner as in Comparative Example 2 except that a 10%

5   polyaniline solution is used instead of the 10%
polypyrrole solution.

(Comparative Example 5)

An enzyme electrode is prepared in the same
manner as in Comparative Example 3 except that a 10%

10   polyaniline solution is used instead of the 10%
polypyrr.ole solution, and 0.63 g of potassium
octacyanotuṅgstate (IV) is used instead of 0.42 g of
the potassium hexacyanoferrate (III).

(Comparative Example 6)

15   An enzyme electrode is prepared in the same
manner as in Example 4 except that the potassium
hexacyanoferrate (III) is not added to the enzyme
solution.

(Comparative Example 7)

20   An enzyme electrode is prepared in the same
manner as in Example 3 except that glucose oxidase
(Aspergillus niger) is used instead of the PQQ-
modified glucose oxidase described in Preparation
Example 14.

25   (Comparative Example 8)

An enzyme electrode is prepared in the same
manner as in Example 4 except that glucose oxidase

(Aspergillus niger) is used instead of the PQQ-
modified glucose oxidase described in Preparation
Example 14.

(Comparative Example 9)

5   An enzyme electrode is prepared in the same
manner as in Example 5 except that glucose oxidase
(Aspergillus niger) is used instead of the PQQ-
modified glucose oxidase described in Preparation
Example 14.

10   (Comparative Example 10)

An enzyme electrode is prepared in the same
manner as in Example 6 except that the potassium
octacyanotungstate (IV) is not added to the enzyme
solution.

15   (Comparative Example 11)

An enzyme electrode is prepared in the same
manner as in Example 7 except that the potassium
octacyanotungstate (IV) is not added to the enzyme
solution.

20   (Comparative Example 12)

An enzyme electrode is prepared in the same
manner as in Example 7 except that D-amino acid
oxidase (swine kidney) is used instead of the
ferrocene-modified amino acid oxidase.

25   (Comparative Example 13)

An enzyme electrode is prepared in the same
manner as in Example 3 except that the osmium complex

polymer described in Preparation Example 8 is used

instead of the osmium complex polymer described in

Preparation Example 6.

(Comparative Example 14)

5   An enzyme electrode is prepared in the same

manner as in Example 4 except that the potassium

hexacyanomanganate (III) is used instead of potassium

hexacyanof errate (III) .

(Comparative Example 15)

10   An enzyme electrode is prepared in the same

manner as in Comparative Example 13 except that the

ferrocene-modified glucose oxidase described in

Preparation Example 15 is used instead of the PQQ-

modified glucose oxidase described in Preparation

15 Example 14.

(Comparative Example 16)

.An enzyme electro.de is prepared in the same

manner as in Example 6 except that the potassium

hexacyanomanganate (III) is used instead of potassium

20 octacyanotungstate (IV) .

(Comparative Example 17)

An enzyme electrode is prepared in the same

manner as in Comparative Example 6 except that

polyvinylpyridine (av mol wt : 150,000) is used

25 instead of the polyvinylimidazole described in

Preparation Example 6.

(Comparative Example 18)

An enzyme electrode is prepared in the same manner as in Comparative Example 8 except that polyvinylpyridine (av mol wt: 150,000) is used instead of the polyvinylimidazole described in

5　Preparation Example 6.

(Comparative Example 19)

An enzyme electrode is prepared in the same manner as in Example 9 except that the potassium octacyanotungstate (IV) is not added to the enzyme

10　solution.

(Comparative Example 20)

An enzyme electrode is prepared in the same manner as in Example 9 except that glucose oxidase (Aspergillus niger) is used instead of the PQQ-

15　modified glucose oxidase described in Preparation Example 14.

(Comparative Example 21)-

An enzyme electrode is prepared in .the same manner as in Example 10 except that glucose oxidase

20　(Aspergillus niger) is used instead of the PQQ- modified glucose oxidase described in Preparation Example 14.

(Comparative Example 22)

An enzyme electrode is prepared in the same

25　manner as in Comparative Example 2 except that foamed nickel alloy (Mitsubishi Materials Corp.; constituting elements: Ni, Cr, Ti, Nb, Al, Mn, Si,

and C; 0.5 mm thick; plated with 0.5μm thick gold;

pore size: 50 μm) is used instead of the gold sheet.

(Comparative Example 23)

An enzyme electrode is prepared in the same

5   manner as in Comparative Example 3 except that foamed

nickel alloy (Mitsubishi Materials Corp.;

constituting elements: Ni, Cr, Ti, Nb, Al, Mn, Si,

and C; 0.5 mm thick; plated with 0.5μm thick gold;

pore size: 50 μm) is used instead of the gold sheet.

10   (Comparative Example 24)

An enzyme electrode is prepared in the same

manner as in Comparative Example 4 except that foamed

nickel alloy (Mitsubishi Materials Corp.;

constituting elements: Ni, Cr, Ti, Nb, Al, Mn, Si,

15   and C; 0.5 mm thick; plated with 0.5μm thick gold;

pore size: 50 μm) is used instead of the gold sheet.

(Comparative Example 25)

An enzyme electrode is prepared in the same

manner as in Comparative Example 5 except that foamed

20   nickel alloy (Mitsubishi Materials Corp.;

constituting elements: Ni, Cr, Ti, Nb, Al, Mn, Si,

and C; 0.5 mm thick; plated with 0.5μm thick gold;

pore size: 50 μm) is used instead of the gold sheet.

(Comparative Example 26)

25   An enzyme electrode is prepared in the same

manner as in Comparative Example 6 except that foamed

nickel alloy (Mitsubishi Materials Corp.;

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constituting elements: Ni, Cr, Ti, Nb, Al, .Mn, Si,

and C; 0.5 mm thick; plated with 0.5μm thick gold;

pore size: 50 μm) is used instead of the gold sheet.

(Comparative Example 27)

5   An enzyme electrode is prepared in the same

manner as in Comparative Example 7 except that foamed

nickel alloy (Mitsubishi Materials Corp.;

constituting elements: Ni, Cr, Ti, Nb, Al, Mn, Si,

and C; 0.5 mm thick; plated with ,0.5μm thick gold;

10   pore size: 50 μm) is used instead of the .gold sheet.

(Comparative Example 28)

An enzyme electrode is prepared in the same

manner as in Comparative Example 8 except that foamed

·nickel alloy (Mitsubishi Materials Corp.;

15   constituting. elements: Ni, Cr, Ti, Nb, Al, Mn, Si,

and C; 0.5 mm thick; plated with 0.5μm thick gold;

pore size: 50 μm) is used instead of the gold sheet.

(Comparative Example 29)

An enzyme electrode is prepared in the same

20   manner as in Comparative Example 9 except that foamed

nickel alloy (Mitsubishi Materials Corp.;

constituting elements: Ni, Cr, Ti, Nb, Al, Mn,. Si,

and C; 0.5 mm thick; plated with 0.5μm thick gold;

pore size: 50 μm) is used instead of the gold sheet-.

25   (Comparative Example 30)

An enzyme electrode is prepared in the same

manner as in Comparative Example 10 except that

foamed nickel alloy (Mitsubishi Materials Corp.;

constituting elements: Ni, Cr, Ti, Nb, Al, Mn, Si,

and C; 0.5 mm thick; plated with 0.5μm thick gold;

pore size: 50 μm) is used instead of the gold sheet.

5   (Comparative Example 31)

.An enzyme electrode is prepared in the same

manner as in Comparative Example 11 except that

foamed nickel alloy (Mitsubishi Materials Corp.;

constituting elements: Ni, Cr, Ti, Nb, Al, Mn,- Si,

10   and C; 0.5 mm thick; plated with 0.5μm thick gold;

pore size: 50 μm) is used instead of the gold sheet.

(Comparative Example 32)

An enzyme electrode is prepared in the same

manner as. in Comparative Example 12 except that

15   foamed nickel alloy (Mitsubishi Materials Corp.;

constituting elements: Ni,. Cr, Ti,. Nb, Al, Mn, Si,

and C; 0.5 mm thick; plated with 0.5μm thick gold;

pore size: 50 μm) is used instead of the gold sheet.

(Comparative Example 33)

20   An enzyme electrode is prepared in the same

manner as in Comparative Example 13 except that

foamed nickel alloy (Mitsubishi Materials Corp.;

constituting elements: Ni, Cr, Ti, Nb, Al, Mn, Si,

and C; 0.5 mm thick; plated with 0.5μm thick gold;

25   pore size: 50 μm) is used instead of the gold sheet.

(Comparative Example 34)

An enzyme electrode is prepared in the same

manner as in Comparative Example 14 except that foamed nickel alloy (Mitsubishi Materials Corp.; constituting elements: Ni, Cr, Ti, Nb, Al, Mn, Si, and C; 0.5 mm thick; plated with 0.5 µm thick gold;

5   pore size: 50 µm) is used instead of the gold sheet.

(Comparative Example 35)

An enzyme electrode is prepared in the same manner as in Comparative Example 15 except that foamed nickel alloy (Mitsubishi Materials Corp.;

10   constituting elements: Ni, Cr, Ti, Nb, Al, Mn, Si, and C; 0.5 mm thick; plated with 0.5 µm thick gold; pore size: 50 µm) is used instead of the gold sheet.

(Comparative Example 36)

An enzyme electrode is prepared in the same

15   manner as in Comparative Example 16 except that foamed nickel alloy (Mitsubishi Materials Corp.; constituting elements: Ni, Cr, Ti, Nb, Al, Mn, Si, and C; 0.5 mm thick; plated with 0.5 µm thick gold; pore size: 50 µm) is used instead of the gold sheet.

20   (Comparative Example 37)

An enzyme electrode is prepared in the same manner as in Comparative Example 17 except that foamed nickel alloy (Mitsubishi Materials Corp.; constituting elements: Ni, Cr, Ti, Nb, Al, Mn, Si,

25   and C; 0.5 mm thick; plated with 0.5 µm thick gold; pore size: 50 µm) is used instead of the gold sheet.

(Comparative Example 38)

An enzyme electrode is prepared in the same manner as in Comparative Example 18 except that foamed nickel alloy (Mitsubishi Materials Corp.; constituting elements: Ni, Cr, Ti, Nb, Al, Mn, Si,

5   and c; 0.5 mm thick; plated with 0.5 μm thick gold; pore size: 50 μm) is used instead of the gold sheet.

(Comparative Example 39)

An enzyme electrode is prepared in the same manner as in Comparative Example 19 except that

10   foamed nickel alloy (Mitsubishi Materials Corp.; constituting elements: Ni, Cr, Ti, Nb, Al, Mn, Si, and c; 0.5 mm thick; plated with 0.5 μm thick gold; pore size: 50 μm) is used instead of the gold sheet.

(Comparative Example 40)

15   An enzyme electrode is prepared in the same manner as in Comparative Example 20 except that foamed nickel alloy (Mitsubishi Materials Corp.; constituting elements: Ni, Cr, Ti, Nb, Al, Mn, Si, and c; 0.5 mm thick; plated with 0.5 μm thick gold;

20   pore size: 50 μm) is used instead of the gold sheet.

(Comparative Example 41)

An enzyme electrode is prepared in the same manner as in Comparative Example 21 except that foamed nickel alloy (Mitsubishi Materials Corp.;

25   constituting elements: Ni, Cr, Ti, Nb, Al, Mn, Si, and c; 0.5 mm thick; plated with 0.5 μm thick gold; pore size:- 50 μm) is used instead of the gold sheet.

(Comparative Example 42)

An enzyme electrode is prepared in the same manner as in Example 6 except that the conductive member constituted of poly (3, 4-ethylenedioxythiphene

5   having numerous voids described in Preparation Example 1 is used instead of the gold sheet.

(Comparative Example 43)

An enzyme electrode is prepared in the same manner as in Example 7 except that the conductive

10   member constituted of poly (3, 4-ethylenedioxythiphene) having numerous voids described in Preparation Example 1 is used instead of the gold sheet.

(Comparative Example 44)

.An enzyme electrode is prepared in the same

15   manner as in Example 6 except that the conductive member constituted of ITO described in Preparation Example 2 is .used instead of the gold sheet.

(Comparative Example 45)

An enzyme electrode is prepared in the same

20   manner as in Example 7 except that the -conductive member constituted of .ITO described in Preparation Example 2 is used instead of the gold sheet.

(Comparative Example 46)

An enzyme electrode is prepared in the same

25   manner as in Example 6 except that a transparent conductive glass sheet constituted of fluorine-doped tin oxide (Asahi Glass Co./ 8Ω/sq) is used instead of

the gold sheet.

(Comparative Example 47)

An enzyme electrode is prepared in the same manner as in Example 7 except that a transparent
5   conductive glass sheet constituted of fluorine-doped tin oxide (Asahi Glass Co.; 8Ω/sq) is- used instead of the gold sheet.

(Comparative Example 48)

An enzyme electrode is prepared in the same
10 manner as in Example 6 except that the conductive member constituted of graphite having numerous voids described in Preparation Example 3 is used instead of the gold sheet. .

(Comparative Example 49)

15 An enzyme electrode is prepared in the same manner as in Example 7 except that the conductive member constituted of graphite having numerous voids described. in Preparation. Example 3 is used instead of the gold sheet.

20 (Example 25)

Sensors are prepared with enzyme electrodes described in Examples 1-24 and Comparative Examples 1-49. A three-electrode cell (Fig. 6) is used which has an enzyme electrode as the working electrode with
25 a leading wire, an Ag/AgCl electrode as the reference electrode, and a platinum wire as the counter electrode. The electrolyte for a glucose oxidase-

employing enzyme electrode is a 20mM phosphate buffer
solution (pH 7.2) containing 15mM glucose and 0.IM
NaCl. The electrolyte for a D-amino acid oxidase-
employing enzyme electrode is a 0.IM pyrophosphate

5   buffer solution (pH 8.5) containing 15mM D-alanine.
In the measurement, the respective electrodes are
connected to a potentiostat (Toho Giken K.K.; Model
2000), and a steady-state- electric current is
recorded by application of a potential of 0.5 V (vs

10   Ag/AgCl) in a nitrogen atmosphere. A water-jecketed
cell is used as the vessel. The measurement
temperature is kept at 37°C by a constant temperature
bath. Tables 1(1) -1(3) show the results.

Table 1(1)

Enzyme electrode	Current density (No substrate) μA/cm²	Current density (Substrate added) μA/cm²
Example 1	4	560
Example 2	7	520
Example 3	7	950
Example 4	7	720
Example 5	4	690
Example 6	7	630
Example 7	2	150
Example 8	1	540
Example 9	8	540
Example 10	9	460
Example 11	51	4100
Example 12	56	3500
Example 13	61	7500
Example 14	59	5200
Example 15	56	5500
Example 16	42	4600
Example 17	13	1100
Example 18	11	3600
Example 19	28	4000
Example 20	69	3700
Example 21	15	4800
Example 22	26	2800
Example 23	16	1500
Example 24	15	1100

Table 1(2)

Enzyme electrode	Current density (No substrate) $\mu A/cm^2$	Current density (Substrate added) $\mu A/cm^2$
Comp.Ex. 1	1	150
Comp.Ex. 2	2	160
Comp.Ex. 3	1	98
Comp.Ex. 4	1	140
Comp.Ex. 5	0	170
Comp.Ex. 6	1	46
Comp.Ex. 7	1	350
Comp.Ex. 8	2	280
Comp.Ex. 9	6	320
Comp.Ex. 10	0	28
Comp.Ex. 11	0	5
Comp.Ex. 12	1	95
Comp.Ex. 13	0	0
Comp.Ex. 14	0	0
Comp.Ex. 15	0	0
Comp.Ex. 16	0	0
Comp.Ex. 17	1	49
Comp.Ex. 18	1	180
Comp.Ex. 19	1	39
Comp.Ex. 20	4	230
Comp.Ex. 21	3	190
Comp.Ex. 22	5	1100
Comp.Ex. 23	8	1400
Comp.Ex. 24	5	950
Comp.Ex. 25	18	1200

Comp.Ex.: Comparative Example

Table 1(3)

Enzyme electrode	Current density (No substrate) $\mu A/cm^2$	Current density (Substrate added) $\mu A/cm^2$
Comp.Ex. 26	3	360
Comp.Ex. 27	36	2400
Comp.Ex. 28	5	1900
Comp.Ex. 29	3	2100
Comp.Ex. 30	3	220
Comp.Ex. 31	0	37
Comp.Ex. 32	10	740
Comp.Ex. 33	0	0
Comp.Ex. 34	0	0
Comp.Ex. 35	0	0
Comp.Ex. 36	0	0
Comp.Ex. 37	4	360
Comp.Ex. 38	14	1400
Comp.Ex. 39	2	280
Comp.Ex. 40	36	1800
Comp.Ex. 41	21	1400
Comp.Ex. 42	2	240
Comp.Ex. 43	18	1700
Comp.Ex. 44	1	140
Comp.Ex. 45	12	1100
Comp.Ex. 46	1	72
Comp.Ex. 47	5	530
Comp.Ex. 48	0	56
Comp.Ex. 49	8	430

Comp.Ex.: Comparative Example

     The sensors which employ an enzyme electrode of Examples 1-24 containing a first mediator for transferring electrons to or from the enzyme and a second mediator for transferring electrons to or from

5 the first mediator, give a higher electric current density than corresponding enzyme electrodesprepared using only one kind of mediator. Presumably, introduction of the first mediator increases the enzyme /mediator electron transfer rate, and the

10 entrapping immobilization increases the density of the immobilized enzyme in the conductive member. On the other hand, the second mediators used in the enzyme electrodes of Comparative Examples 13-16 have redox potentials (reduction-oxidation potentials) of

15 -0.19 V (vs Ag/AgCl, Examples 13, 15) and -1.02 V (vs Ag/AgCl, Examples 14, 16) according to literature. These redox potentials, (reduction-oxidation potentials) are more negative than that of the corresponding first mediators (-0.14 V vs Ag/AgCl at

20 the most negative) . Therefore, in these Comparative Examples, electron transfer does not occur between the first mediator and the second mediator, resulting in a low current density. Thus, the enzyme electrode having the first mediator for transfer of electrons

25 to or from the enzyme and the second mediator for trans.fer of electrons to or from the first mediator can improve the sensitivity of the sensor.

(Example   2 6)

.Fuel cells are prepared with enzyme electrodes
described in Examples 1-24 and Comparative Examples
1-49. The cell has a two-electrode constitution (Fig..

5   7) which has an enzyme electrode with a leading wire.
as the working electrode, an enzyme electrode
described in Preparation Example 17 as the counter
electrode, and a porous polypropylene film- (20 μm
thick) held between the electrodes. The electrolyte

10   is a 0.2M citrate buffer solution (pH 5.0). The
anode fuel is 15 mM glucose for .glucose oxidase-
employing electrodes, · and 15mM D-alanine for D-amino
acid oxidase-employing electrodes: the cathode fuel
is saturated oxygen. In the measurement, with the

15   respective electrodes connected to a potentiostat
(Toho Giken K .K.; Model 2000), the voltage-current
characteristics are derived by changing the voltage
from -1.2 V to 0.1 V at a rate of 1 mV/sec. The
measurement vessel is a.water-jacketed' cell, and the

20   measurement temperature is kept at 37°C by a constant
temperature bath. Tables 2(1) -2(3) ·show the results.

Table 2(1)

Anode	Cathode	Short-circuit current density μA/cm²	Maximum output μW/cm²
Example 1	Prepn.Ex. 17	450	53
Example 2	Prepn.Ex. 17	420	17
Example 3	Prepn.Ex. 17	760	231
Example 4	Prepn.Ex. 17	580	76
Example 5	Prepn.Ex. 17	550	22
Example 6	Prepn.Ex. 17	500	20
Example 7	Prepn.Ex. 17	120	5
Example 8	Prepn.Ex. 17	430	56
Example 9	Prepn.Ex. 17	430	18
Example 10	Prepn.Ex. 17	370	78
Example 11	Prepn.Ex. 17	3000	317
Example 12	Prepn.Ex. 17	2800	100
Example 13	Prepn.Ex. 17	3000	818
Example 14	Prepn.Ex. 17	3000	320
Example 15	Prepn.Ex. 17	3000	111
Example 16	Prepn.Ex. 17	3000	112
Example 17	Prepn.Ex. 17	870	32
Example 18	Prepn.Ex. 17	2900	324
Example 19	Prepn.Ex. 17	3000	112
Example 20	Prepn.Ex. 17	2900	555
Example 21	Prepn.Ex. 17	3000	403
Example 22	Prepn.Ex. 17	2300	557
Example 23	Prepn.Ex. 17	1200	237
Example 24	Prepn.Ex. 17	870	49

Prepn.Ex.: Preparation Example

Table 2 (2)

Anode	Cathode	Short-circuit current density $\mu A/cm^2$	Maximum output $\mu W/cm^2$
Comp.Ex. 1	Prepn.Ex. 17	120	54
Comp.Ex. 2	Prepn.Ex. 17	130	57
Comp.Ex. 3	Prepn.Ex. 17	79	10
Comp.Ex. 4	Prepn.Ex. 17	110	49
Comp.Ex. 5	Prepn.Ex. 17	140	6
Comp.Ex. 6	Prepn.Ex. 17	37	16
Comp.Ex. 7	Prepn.Ex. 17	280	84
Comp.Ex. 8	Prepn.Ex. 17	220	26
Comp.Ex. 9	Prepn.Ex. 17	260	11
Comp.Ex. 10	Prepn.Ex. 17	22	2
Comp.Ex. 11	Prepn.Ex. 17	4	0
Comp.Ex. 12	Prepn.Ex. 17	76	3
Comp.Ex. 13	Prepn.Ex. 17	0	0
Comp.Ex. 14	Prepn.Ex. 17	0	0
Comp.Ex. 15	Prepn.Ex. 17	0	0
Comp.Ex. 16	Prepn.Ex. 17	0	0
Comp.Ex. 17	Prepn.Ex. 17	39	18
Comp.Ex. 18	Prepn.Ex. 17	150	18
Comp.Ex. 19	Prepn.Ex. 17	31	14
Comp.Ex. 20	Prepn.Ex. 17	180	8
Comp.Ex. 21	Prepn.Ex. 17	150	32
Comp.Ex. 22	Prepn.Ex. 17	890	354
Comp.Ex. 23	Prepn.Ex. 17	1100	120
Comp.Ex. 24	Prepn.Ex. 17	760	315
Comp.Ex. 25	Prepn.Ex. 17	960	36

Comp. Ex. : Comparative Example

Prepn.Ex. : Preparation Example

Table 2 (3)

Anode	Cathode	Short-circuit current density μA/cm^2	Maximum output μW/cm^2
Comp.Ex. 26	Prepn.Ex. 17	290	119
Comp.Ex. 27	Prepn.Ex. 17	1900	523
Comp.Ex. 28	Prepn.Ex. 17	1500	164
Comp.Ex. 29	Prepn.Ex. 17	1700	60
Comp.Ex. 30	Prepn.Ex. 17	180	12
Comp.Ex. 31	Prepn.Ex. 17	29	2
Comp.Ex. 32	Prepn.Ex. 17	590	22
Comp.Ex. 33	Prepn.Ex. 17	0	0
Comp.Ex. 34	Prepn.Ex. 17	0	0
Comp.Ex. 35	Prepn.Ex. 17	0	0
Comp.Ex. 36	Prepn.Ex. 17	0	0
Comp.Ex. 37	Prepn.Ex. 17	290	114
Comp.Ex. 38	Prepn.Ex. 17	1200	132
Comp.Ex. 39	Prepn.Ex. 17	230	96
Comp.Ex. 40	Prepn.Ex. 17	1400	56
Comp.Ex. 41	Prepn.Ex. 17	1100	194
Comp.Ex. 42	Prepn.Ex. 17	190	41
Comp.Ex. 43	Prepn.Ex. 17	1400	190
Comp.Ex. 44	Prepn.Ex. 17	120	39
Comp.Ex. 45	Prepn.Ex. 17	850	208
Comp.Ex. 46	Prepn.Ex. 17	58	17
Comp.Ex. 47	Prepn.Ex. 17	430	87
Comp.Ex. 48	Prepn.Ex. 17	45	4
Comp.Ex. 49	Prepn.Ex. 17	340	18

Comp.Ex.: Comparative Example

Prepn.Ex.: Preparation Example

The fuel cells, which employ an enzyme electrode of Examples 1-24 -containing a first mediator for transferring electrons to or from the enzyme and a second mediator for transferring

5   electrons to or from the first mediator, give a higher short-circuit current density than corresponding enzyme electrodes prepared using only one kind of mediator. Presumably, introduction of the first mediator increases the enzyme/mediator

10   electron transfer rate, and the entrapping immobilization increases the density of the immobilized enzyme in the conductive member. On the other hand, the- second mediators used in the enzyme electrodes of Comparative Examples 13-16 have redox

15   potentials (reduction-oxidation potentials) of -0.19 V (vs Ag/AgCl, Comparative Examples 13, 15) and -1.02 V (vs Ag/AgCl, Cpmparative Examples 14, 16) according to literature. These redox potentials -(reduction-oxidation potentials) are more negative than that of

20   the corresponding first mediators (-0.14 V vs Ag/AgCL at the most negative) . Therefore, in these Comparative Examples, electron transfer does not occur between the first mediator and the second mediator, resulting in a low current density. In

25   some of the fuel cells of Examples, the maximum output is lower than that of the corresponding fuel cells of Comparative Examples without the mediators

owing to a voltage loss by introduction of plural
mediators. However,- as a whole, the fuel cells
employing the enzyme electrodes of Examples give
higher output than the corresponding to the fuel

5   cells of Comparative Examples without the mediators.
Thus, ·the enzyme electrode having the first mediator
for transfer of electrons to or from the enzyme and
the second mediator for transfer of electrons to or -
from the first mediator can improve the output of the

10 fuel cells.

(Example 27)

Electrochemical reactors are prepared with
enzyme electrodes described in Examples 1-24 and
Comparative Examples 1-49. A three-electrode cell

15 (Fig. 6) is used which has an enzyme electrode as the
working electrode, an Ag/AgCl -electrode as the
reference electrode, and a platinum wire as the
counter electrode. The electrolyte solution is a
20mM phosphate buffer solution (pH 7.2) containing

20 0.1M NaCl, 10mM glucose, and 10mM D-alanine. In the
measurement, a potential of 0.4 V vs Ag/AgCl is
-applied for 100 minutes in a water-jecketed cell in a
nitrogen atmosphere. The formed product is
quantitatively determined by high-speed liquid

25 chromatography. Tables 3(1) -3 (3) show the results. ,

Table 3(1)

Enzyme electrode	Reaction charge mC	Detected substance	Detected quantity μmol
Example 1	3200	Gluconolactone	32
Example 2	2900	Gluconolactone	30
Example 3	5400	Gluconolactone	56
Example 4	4000	Gluconolactone	41
Example 5	4100	Gluconolactone	41
Example 6	3400	Gluconolactone	37
Example 7	800	Pyruvic acid	8
Example 8	2900	Gluconolactone	32
Example 9	3200	Gluconolactone	34
Example 10	2600	Gluconolactone	27
Example 11	25000	Gluconolactone	250
Example 12	21000	Gluconolactone	210
Example 13	44000	Gluconolactone	450
Example 14	29000	Gluconolactone	310
Example 15	30000	Gluconolactone	300
Example 16	26000	Gluconolactone	280
Example 17	6300	Pyruvic acid	64
Example 18	20000	Gluconolactone	200
Example 19	22000	Gluconolactone	230
Example 20	20000	Gluconolactone	210
Example 21	28000	Gluconolactone	290
Example 22	15000	Gluconolactone	170
Example 23	7900	Gluconolactone	86
Example 24	6300	Gluconolactone	65

Table 3(2)

Enzyme electrode	Reaction charge mC	Detected substance	Detected quantity μmol
Comp.Ex. 1	830	Gluconolactone	9
Comp.Ex. 2	880	Gluconolactone	9
Comp.Ex. 3	550	Gluconolactone	6
Comp.Ex. 4	770	Gluconolactone	8
Comp.Ex. 5	940	Gluconolactone	10
Comp.Ex. 6	260	Gluconolactone	3
Comp.Ex. 7	2000	Gluconolactone	21
Comp.Ex. 8	1600	Gluconolactone	16
Comp.Ex. 9	1900	Gluconolactone	21
Comp.Ex. 10	160	Gluconolactone	2
Comp.Ex. 11	26	Pyruvic acid	0
Comp.Ex. 12	540	Pyruvic acid	6
Comp.Ex. 13	0	Gluconolactone	0
Comp.Ex. 14	0	Gluconolactone	0
Comp.Ex. 15	0	Gluconolactone	0
Comp.Ex. 16	0	Gluconolactone	0
Comp.Ex. 17	270	Gluconolactone	3
Comp.Ex. 18	1000	Gluconolactone	11
Comp.Ex. 19	210	Gluconolactone	2
Comp.Ex. 20	1300	Gluconolactone	13
Comp.Ex. 21	1100	Gluconolactone	12
Comp.Ex. 22	6700	Gluconolactone	71
Comp.Ex. 23	8100	Gluconolactone	88
Comp.Ex. 24	5200	Gluconolactone	54
Comp.Ex. 25	7100	Gluconolactone	77

Comp. Ex.: Comparative Example

Table 3(3)

Enzyme electrode	Reaction charge mC	Detected substance	Detected quantity μmol
Comp.Ex. 26	2000	Gluconolactone	22
Comp.Ex. 27	14000	Gluconolactone	150
Comp.Ex. 28	11000	Gluconolactone	110
Comp.Ex. 29	12000	Gluconolactone	120
Comp.Ex. 30	1300	Gluconolactone	13
Comp.Ex. 31	220	Pyruvic acid	2
Comp.Ex. 32	4000	Pyruvic acid	41
Comp.Ex. 33	0	Gluconolactone	0
Comp.Ex. 34	0	Gluconolactone	0
Comp.Ex. 35	0	Gluconolactone	0
Comp.Ex. 36	0	Gluconolactone	0
Comp.Ex. 37	2100	Gluconolactone	22
Comp.Ex. 38	8100	Gluconolactone	84
Comp.Ex. 39	1600	Gluconolactone	16
Comp.Ex. 40	10000	Gluconolactone	110
Comp.Ex. 41	8000	Gluconolactone	86
Comp.Ex. 42	1500	Gluconolactone	15
Comp.Ex. 43	9900	Gluconolactone	98
Comp.Ex. 44	850	Gluconolactone	9
Comp.Ex. 45	6000	Gluconolactone	64
Comp.Ex. 46	410	Gluconolactone	4
Comp.Ex. 47	3100	Gluconolactone	32
Comp.Ex. 48	310	Gluconolactone	3
Comp.Ex. 49	2500	Gluconolactone	27

Comp.Ex.: Comparative Example

After the reaction, except the reactor
employing the enzyme electrode of- Comparative
Examples 13-16, from the electrolytic solution of the
electrochemical reactor having an enzyme electrode

5   having a glucose-specific enzyme, gluconolactone is
detected without detecting pyruvic acid, whereas from
the electrolytic solution of the electrochemical
reactor having an enzyme electrode having a D-
alanine-specific enzyme, pyruvic acid is detected

10   without detecting gluconolactone. Thus in any of the
enzyme electrode-employing reactor, the reaction
proceeds by selection of the substrate. On the other
hand, the second mediators used in the enzyme
electrodes of Comparative Examples 13-16 have redox

15   potentials (reduction-oxidation potentials) of -0.19
V (vs Ag/AgCl, Comparative Examples 13, 15) and -1.02
V (vs Ag/AgCl, Comparative Examples 14, 16) according
to literature. These redox potentials (reduction-
oxidation potentials) are more negative than that of

20   the corresponding first mediators (-0.14 V vs Ag/AgCl
at the most negative). Therefore, in these
Comparative Examples electron transfer does not occur
between the first mediator and the second mediator,
resulting in a low current density. Thus, the enzyme

25   electrode having the first mediator for transfer of
electrons to or from the enzyme and the second
mediator for transfer of electrons to or from the

first mediator can increase the reaction rate and increase of the sensitivity of the electrochemical reaction.

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    This application claims priority from Japanese Patent Application No. 2004-216377 filed on July 23, 2004, which is hereby incorporated by reference herein.